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L2: Entry 42 of 60

File: USPT

Mar 14, 1995

DOCUMENT-IDENTIFIER: US 5397694 A  
TITLE: Protease inhibitor

Abstract Paragraph Right (1):

This invention relates to a novel protease-inhibitor,--which we called GELIN--and to pharmaceutical and cosmetic preparations thereof, containing this compound. GELIN is an inhibitor of human and porcine leucocyte elastase and chymotrypsin. GELIN has specific antibiotic properties. It also relates to the novel use of EGLIN, another chymotrypsin-inhibitor in cosmetic preparations.

Brief Summary Paragraph Right (2):

Gelin is an inhibitor of human and porcine leucocyte-elastase and chymotrypsin. Gelin has specific antibiotic properties. It also relates to the novel use of Eglin, another chymotrypsin-inhibitor in cosmetic preparations.

Brief Summary Paragraph Right (3):

Several diseases, like emphysema, arthritis, gingivitis, periodontitis and other inflammatory conditions occur due to tissue destruction caused by the enzyme Elastase. Elastases are the only serine proteases which are capable of solubilising fibrous proteins like elastin and collagen. They are chiefly present in pancreas and in the azurophilic granules of neutrophil leucocytes. Under normal physiological conditions, the proteolytic activity of the enzyme is kept under check by the excess of inhibitors present in plasma and other secretions. However, under diseased state, local deficiency of inhibitor leads to an imbalance resulting in tissue destruction, the underlying cause of various inflammatory conditions.

Brief Summary Paragraph Right (4):

As an example this situation is described for gingivitis. Disorders of neutrophil-functions are often associated with abnormalities and conditions of the host, e.g.: diabetes mellitus, Down's syndrome, ichthyosis, rheumatoid arthritis, cyclic neutropenia, agranulocytosis, Chediak-Higashi syndrome. (Cianciola, L. J., et al., Nature (1977), 265:445-447; Cohen, W. D., et al., J. Period (1961), 32:159-168). Polymorphonuclear (PMN)-derived neutral proteases and/or bacterial toxins directly or indirectly attach supporting tissues in the dento-gingival area so as to cause inflammation (Janoff, A., J. Am. Path. (1972), 68:538-623; Weiss, S. J., New Engl. J. Med. (1989), 320-6:365-376; Henson, P. M., et al., J. Clin. Invest. (1987), 79:669-674; Campbell, E. J., et al., J. Clin. Invest. (1982), 70:845-852; Lehrer, R. I., et al., Ann. Int. Med. (1989), 109:127-142). Gingival crevicular fluid from inflamed gingival tissues contains high levels of hydrolytic enzymes. (Cimasoni, G., Monographs in Oral Science, Vol. 12.ed., H. M. Myers, Philadelphia, Pa., Karger (1983)). Oxygen radicals have both bacteriostatic as well as tissue-destructing activities. (Schalkwijk, J., Thesis, Nijmegen Holland (1986)). This tissue-destructing activity is at discussion. (Weiss, S. J., New Engl. J. Med. (1989), 320-6:365-376; Henson, P. M., et al., J. Clin. Invest. (1987), 79:669-674; Dakin, H. D., Br. Med. J. (1915), 2:318-320). Chlorinated oxidating agents exert strong microbicidal activity (Clark, P. A., et al., Infect. Immun. (1986), 53-2:252-256) and are so as to be only suitable for use in a simple in-vitro buffer system (Dakin, H. D., Br. Med. J. (1916), 1:852-854; Dakin, H. D., JAMA (1917), 69:27-30). Already in 1917 the use of synthetic chloramines was recommended for irrigation of wounds. Still now, it remains dubious, whether the final oxidizing agent is HOCl or the derivative chloramine. (Weiss, S. J., et al., Science (1983), 222:625-628). While hydrolising, lysosomal enzymes, derived from PMN degranulation, are considered a threat to various tissue-constituents, (Weiss, S. J., New Engl. J. Med. (1989), 320-6:365-376; Campbell, E. J., et al., J.

Clin. Invest. (1982), 70:845-852) natural serum protease-inhibitors (alpha 1 protease inhibitor and alpha macroglobulin) are at large inactivated by its myeloperoxidase-oxydizing system. (Weiss, S. J., New Engl. J. Med. (1989), 320-6:365-376; Kramps, J. A., et al., Clin. Science (1988), 75:53-62) Bacterial derived toxins (low-molecular weight metabolic products, glycoproteins, lipopolysaccharides, proteases ) are reported to initiate host tissue- and cell destruction as well as immune-cell activation. (Cimasoni, G., Monographs in Oral Science, Vol. 12.ed., H. M. Myers, Philadelphia, Pa., Karger (1983); Bom-vNoorloos et al., J. Clin. Periodontal. (1989), 16:412-418; Curtis, M. A., et al., J. Clin. Periodontal. (1989), 16:1-11; Carpenter, A. B., et al., Inf. Immun. (1984); 43-1:326-336). Some microorganisms are able to inactivate the human serum protease-inhibitors. (Carlsson, J., et al., Infect. Immun. (1984), 43-2:644-648; Morihara, M., et al., Infect. Immun. (1979); 24:188-193). Consequently, a potent inhibitor of elastase might prove to be a useful therapeutic tool to combat such diseases.

Brief Summary Paragraph Right (5):

Studies have shown that the salivary glands of leeches contain a potent inhibitor to the enzyme elastase. In the leech species Hirudo medicinalis, apart from the thrombin inhibitor hirudin, an inhibitor to the enzymes chymotrypsin and elastase has also been observed. It has been named Eglin and has been purified and well characterized (Seemuller U., et al., Meth. Enzymol. (1981), 804-816). Goldstein et al, (Goldstein, A. M., et al., Comp. Biochem. Physiol. (1986); 84B:117-124) have reported the presence of Eglin in three different species of North American leeches. However, to our knowledge, the elastase-inhibitor from other species of leeches studied so far has similar biochemical properties.

Brief Summary Paragraph Right (6):

In the present study, during the purification of anti-thrombin from a leech species Hirudinaria manillensis, we have isolated unintentionally, a novel inhibitor which has potent anti-chymotrypsin and anti-elastase activity. The results obtained so far indicate that this inhibitor is significantly different from Eglin, and has been named "Gelin".

Brief Summary Paragraph Right (7):

Gelin was found during experiments for purification of proteins from leech species Hirudinaria manillensis. During the purification of these proteins, inhibitory activity towards chymotrypsin and elastase was observed in certain fractions, and these were studied for comparison with eglin and hirudin. The leech-derived elastase/chymotrypsin inhibitor, according to part of this invention (and corresponding DNA sequences, which can be extrapolated therefrom, or a corresponding synthetic polypeptide is non-homologous with Eglin, a known elastase/chymotrypsin inhibitor, of which it is known to be present in the medicinal leech Hirudo medicinalis and is described by: Seemuller et al.: Eglin: "elastase-catheps in G-inhibitor from leeches"; 1981: Meth.Enzymol.: 80:804-816.

Brief Summary Paragraph Right (8):

Furthermore, comparisons with hirudin (Dodt et al.; FEBS 165,:180-184) and other known structures lead to the conclusion that the structure of Gelin is unique and very different from that of Eglin.

Brief Summary Paragraph Right (10):

The other part of this invention relates to the novel use of Eglin in cosmetic preparations, such as, amongst others: mouth rinses, toothpastes, skin creams.

Drawing Description Paragraph Right (12):

FIG. 11A is the CD spectrum of gelin. FIG. 11B is the CD spectrum of eglin.

Drawing Description Paragraph Right (14):

The concentrated product was applied to a CM Sepharose column, equilibrated with 50 mM sodium acetate pH 6.0. The eluate (material that did not bind to the column) was collected as one large fraction and assayed for anti-chymotrypsin and anti-elastase activity. The eluate which contained the active ingredient was concentrated to 400 ml by ultrafiltration using a 10,000 molecular weight cut off filter.

Drawing Description Paragraph Right (16):

After washing, the bound material was eluted with a stepwise gradient of 0.1-0.4M NaCl in equilibration buffer and each of the eluting peaks was collected as a separate fraction for measurement of anti-chymotrypsin and anti-elastase activity. The active peak was desalted by dialysis overnight against distilled water.

Drawing Description Paragraph Right (17):

The partially purified product was further purified by anion exchange chromatography on Q-Sepharose, pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The column was developed at a flow rate of 20 ml/min and the bound material was eluted with a linear gradient of 0-1M NaCl in equilibration buffer. The absorbance and the elastase inhibitory activity of the eluate was recorded. The active fraction was pooled and concentrated by ultrafiltration using a 10,000 molecular weight cut off filter.

Drawing Description Paragraph Right (19):

Partially purified sample was reconstituted and applied in aliquots to a reverse phase microbore Aquapore C8 column, equilibrated in 0.1% TFA. The bound material was eluted with 0-40% linear gradient of 60% CH.sub.3 CN+0.09% TFA over 10 minutes and 40-100% over 20 minutes. Each peak was collected as a separate fraction and checked for anti-elastase activity. (FIG. 5). The active peak was lyophilized and used for further studies.

Drawing Description Paragraph Right (21):

The elastase inhibitory activity of Gelin was measured spectrophotometrically by measuring the inhibition of the release of p-nitroaniline group from the synthetic substrate N-succinyl (Ala).sub.3 -p-nitroanilide (SAAAP) catalyzed by pancreatic elastase. One inhibitory unit (IU) of activity is defined as the amount of Gelin necessary to inhibit the hydrolysis of 1 .mu. mole of SAAAP/min at pH 8.3 and 25.degree. C.

Drawing Description Paragraph Right (22):

The assay consists of incubating different amounts of Gelin with a known amount of pancreatic elastase in 0.1M Tris/HCl buffer, pH 8.3, containing 1M NaCl for 5 min at 25.degree. C. The reaction is started by the addition of the chromogenic substrate and the absorbance at 405 nm monitored with time. A control reaction, in the absence of Gelin, is carried out under identical conditions. From absorbance change per min and using molar extinction coefficient of 10,500 M.sup.-1 cm.sup.-1, the activity of Gelin can be calculated.

Drawing Description Paragraph Right (25):

The isoelectric point of Gelin was found to be about 4.6, as compared to the published report of 6.45 for Eglin C and 6.6 for Eglin B. (The two Eglins differ by one amino acid; histidine in Eglin B for tyrosine in Eglin C.).

Drawing Description Paragraph Right (26):

The molecular weight of Gelin was determined by SDS-polyacrylamide gel electrophoresis as described by Laemmli. (23). In both 16% and 20% homogeneous gel, purified Gelin migrated as a band just below 14.4 kDa under reducing conditions (FIG. 7A). Molecular weight markers used under identical conditions were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and lactalbumin (14.4 kDa). However, when Gelin was analysed by SDS-PAGE in the presence of 8M urea, according to the method of Swank & Munkres (24), it was found that the sample was difficult to visualise when stained with Coomassie blue, but on silver staining, there was some indication that Gelin had a mobility which corresponded to a molecular weight of about 21-25 kDa. A low molecular weight "Electran" reference suitable for determination of molecular weight of small proteins and peptides by Swank & Munkres' method was used, as shown in FIG. 7B. This discrepancy in molecular weight by two different methods cannot be explained at present. (Eglin has a molecular weight of 8.1 kDa).

Drawing Description Paragraph Right (27):

The inhibitory activity of Gelin was compared against the following serine proteases: elastase, cathepsin G, chymotrypsin, trypsin and thrombin, using assay methods with chromogenic substrates. The details of the assay conditions used for each enzyme are outlined in Table 1. In brief, the fixed amount of enzyme is incubated with different

concentrations of Gelin in the appropriate buffer at 37.degree. C. for 5 min. The reaction is started by the addition of the substrate and the increase in absorbance is monitored at 405 nm. The initial rate of the control assay without the inhibitor was taken as 100% for each enzyme. From the data obtained the molar concentration of Gelin required to inhibit 50% of the enzyme activity (IC<sub>50</sub>) was calculated. The results indicate that Gelin is a potent inhibitor towards chymotrypsin, cathepsin G and elastase, with little activity towards trypsin and thrombin. The IC<sub>50</sub> values calculated were 0.13, 0.25, 0.32, and 20.4 moles of Gelin/mole of chymotrypsin, cathepsin G, elastase and trypsin, respectively.

Drawing Description Paragraph Right (28):

Purified gelin was hydrolysed with gaseous ARISTAR HCl under vacuum at 110.degree. C. for 24 and 48 hrs. The hydrolysed mixture was analyzed for amino acid composition on an Amino Chrome system. For comparison with eglin c, a molecular weight of 8100 daltons was used for gelin for the quantification of the liberated amino acids. The results, as shown in Table 2, indicate that the composition is quite different for both the inhibitors. In particular, the presence of significant amounts of aspartic acid (+asparagine) and alanine, absence of histidine and the presence of isoleucine in gelin, compared to eglin c.

Drawing Description Paragraph Right (29):

CD spectrum of anti-elastase was obtained using 0.02 cm pathlength cell in 0.1% TFA at room temperature. This spectrum was compared with that obtained with rec-eglin (FIGS. 11A and 11B), the latter was a gift from Ciba Geigy, Basel. The data, when evaluated by CONTIN analysis, indicate that the tertiary structure of gelin has no helix, 58% beta sheets and 42% non-ordered structures, compared to 19% helix, 56% beta sheets and 25% non-ordered structures in eglin c. Thus the elastase inhibitor from two different species of leeches are markedly different and further supports the differences observed in their N-terminal amino acid sequences (see below).

Drawing Description Paragraph Right (30):

N-terminal amino acid sequence of purified anti-elastase was determined and this resulted in a single sequence upto residue 29. In order to confirm the presence of cysteine residues in the sequence, purified sample was reduced with dithiothreitol and the cysteine derivatized to pyridyethyl cysteine by reaction with 4-vinyl pyridine. Amino terminal sequence analysis of this derivatized sample was repeated and the results obtained are shown in Table 3. Comparison of this partial sequence with the published data of eglin shows that this inhibitor is markedly different in its primary structure.

Drawing Description Paragraph Right (46):

Gelin is a novel protease inhibitor derived from the leech. The basis of the assay involves the inhibition of elastase activity on the synthetic chromogenic substrate SAAAP. Elastase activity can be measured spectrophotometrically (at 405 nm) by monitoring the release of a coloured p-nitroaniline group liberated during substrate digestion. The reduction in elastase activity in the presence of gelin is related to the inhibitory activity.

Drawing Description Paragraph Right (47):

One unit of elastase activity (U) will hydrolyse 1 umole of SAAAP/min at pH 8.3, 25.degree. C. One unit of inhibitory activity (IU) will reduce the enzyme catalysed hydrolysis by 1 umole of SAAAP/min at pH8.3, 25.degree. C.

STANDARD OPERATING CODE PAGE PROCEDURE GEL 2 2

GELIN ASSAY

MATERIALS Chemicals Supplier

Elastase Sigma E1250 SAAAP: N-Succinyl-L(-Alanine)3- Calbiochem 573459 p-Nitroanilide Acetic Acid - Glacial BDH 10001 NaCl Sigma S9625 Tris Sigma T1503 HCl BDH 10307 Gelin In house preparation Apparatus 96 'U' well microtitre plate Midland Laboratories Titertek Uniskan II Microtitre Flow Laboratories Plate Reader (equipped with 405 nm light filter)

STANDARD OPERATING CODE PAGE PROCEDURE GEL 2 3

GELIN ASSAY

Drawing Description Paragraph Right (52):

The molar extinction coefficient for the coloured product may be determined experimentally by incubating a known quantity of substrate with Elastase until the

reaction is complete (i.e. no further colour change).

Drawing Description Paragraph Right (75):

The important clinical very significant reduction in PBI as tested points towards the protease-inhibiting mechanism, which inhibits PMN-derived neutral proteases (elastase, cath G.) to proteolyze gingival substrates. The less important, but significant reduction in plaque accumulation is surprising, although welcome. This effect is probably due to the fact that the inhibition of proteolysis of supporting tissues reduces the quantity of nutrients necessary for the viability of periodontopathic micro-organisms in dental plaque.

Drawing Description Paragraph Right (78):

Hirudinaria manillensis leeches were carefully dissected into three portions: head, body and gut lining. Each component was well washed with distilled water to remove contaminants (e.g. blood). Each portion was dehydrated in ethanol and the extract assayed for inhibition of elastase.

Drawing Description Paragraph Type 0 (13):

2. Elastase Solution

Drawing Description Paragraph Type 0 (24):

2. Elastase Solution

Drawing Description Paragraph Type 1 (13):

40 ug/ml Elastase in H.sub.2 O

Drawing Description Paragraph Type 1 (18):

50 ul Elastase solution

Detailed Description Paragraph Right (4):

Gelin or Eglin (50 .mu.l, 10 .mu.g/ml in dH.sub.2 O) was incubated with hydrogen peroxide (30%, 50 .mu.l in dH.sub.2 O) for 5 min. at 37.degree. C. An aliquot (50 .mu.l) was removed and assayed for inhibitory activity versus chymotrypsin.

Detailed Description Paragraph Right (6):

Under the conditions described the inhibitory action of Eglin is unaffected. For Gelin the inhibitin action appears to be minimally effected; (Compare tubes 1 and 2), and corresponds to 8%.

Detailed Description Paragraph Right (7):

Isolates of Aeromonas hydrophila were recovered from engorged leeches. Subsequent identification of the protease profile showed them to be elastase positive utilizing an assay described by Shotts et al. (Extracellular proteolytic activity of Aeromonas hydrophila complex. Fish Pathology (1985), 20:37-44). A vial of Gelin containing 1 mg was reconstituted in 1 ml and subsequent dilutions made from 10.sup.-1 -10.sup.-6. A selected culture of A.hydrophila was inoculated on the previously described assay medium so as to achieve a "lawn". A "penicillin" assay cylinder was placed at the center of the plates. One-tenth ml (0.1 ml) of the diluted Gelin was introduced into the respective cylinder and the plates were incubated for 10 days at 25.degree. C. Findings indicated a blockage of elastase activity within the diffusion zone of the Gelin with a slight zone of elastase activity beyond. This was noted to occur at undiluted, 10.sup.-1, 10.sup.-2, and 10.sup.-3.

Detailed Description Paragraph Right (21):

The examples mentioned for both toothpaste preparations as well as the mouth preparation are similar but with the replacement of GELIN by Eglin.

Detailed Description Paragraph Right (22):

The cosmetic application of Eglin in cosmetic creams is similar to the description of the preparation of a pharmaceutical composition, but for the replacement of GELIN by Eglin. Furthermore, this is used uniquely for cosmetic uses under current law.

Detailed Description Paragraph Left (6):

The Use of Eglin in cosmetic preparations, a.o. toothpastes

Detailed Description Paragraph Table (2):

Results: 1 2 3 4 5 6 7 8 9									
50	1	+	+	-	-	-	Gelin	50	1
+	-	+	-	+	-	+	H.sub.2	O.sub.2	Abs.
.405	.085	.060	.640	.656	.060	.059	.563	.611	
.055	(n = 3)	(.+-SD)	.006	.001	.019	.007	.003	.002	.031
			.010	.057					

Detailed Description Paragraph Table (5):

TABLE 1

Assay conditions used to determine the inhibitory potency of gelin towards various serine proteases Enzyme Substrate Enzyme Buffer Substrate conc. conc.

0.1M Hepes Succ--Ala--Ala--Pro--Phe--pNA 16 nM 2.0 mM pH 7.4 Trypsin 0.1M Tris/HCl Cathepsin G  
 Bz--Arg--pNA 50 nM 0.8 mM pH 8.0 Chymotrypsin 0.1M Tris/HCl +  
 MeO--Suc--Arg--Pro--Tyr--pNA 16 nM 2.4 mM 0.96M NaCl pH 8.3 Elastase 0.1M Tris/HCl +  
 Succ--Ala--Ala--Ala 330 nM 0.55 mM 0.05% Triton pNA X-100, pH 8.3 Thrombin 0.1M  
 Tris/HCl + Phe--Pip--Arg--pNA 5 nM 0.08 mM 0.3M NaCl pH 8.4

Detailed Description Paragraph Table (6):

TABLE 2

## COMPARISON OF THE AMINO ACID

COMPOSITION OF GELIN AND EGLIN Purified Gelin was hydrolysed with 6N HCl for 24 and 48 hrs and analysed for amino acid composition. The values calculated are based on an assumed molecular weight of 8100 daltons Gelin 24 hr 48 hr Amino acid moles/mole moles/mole Eglin c\* Asp (+ Asn) 14-15 19 7 Glu (+ Gln) 6 9 7 Ser 4 3-4 3 Thr 2-3 3 5 Gly 8-9 7 5 Ala 7-8 7 1 Arg 2 2 4 Pro 6 4-5 6 Val 11-12 8 11 Met 1 2 Ile 2-3 2 0 Leu 4-5 3-4 5 Phe 2 2 5 Cys Lys 5-6 5 2 His 0 0 3 Tyr 1 0 5-6 Trp N.D. N.D. 0-1 N-terminus Val Val Thr Total 75-83 76-79 69-71

\*Values obtained from ref (1) N.D. = not

determined

Detailed Description Paragraph Table (7):

TABLE 3

## COMPARISON OF THE N-TERMINAL SEQUENCE OF GELIN AND EGLIN

Gelin ValAspGluLys AlaGluValThrAsp GlyLeuCysGlyAspTrp ##STR1## GelinThrCysSerGlyAlaGln ValXaaGlnAsnAspAlaAlaVal

## CLAIMS:

3. A polypeptide according to claim 1, wherein the polypeptide has an isoelectric point of about 4.6 and substantially retains its anti-elastase activity after incubation at 100.degree. C. for 30 minutes.

4. A pharmaceutical formulation comprising a polypeptide according to claim 1, in an elastase inhibitory amount, together with a pharmaceutically acceptable carrier, diluent or excipient.

13. A method of inhibiting elastase activity of a bacterial source, which comprises contacting said source with a polypeptide according to claim 1 in an amount sufficient to inhibit elastase activity.

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L2: Entry 45 of 60

File: USPT

Jan 11, 1994

DOCUMENT-IDENTIFIER: US 5278285 A

TITLE: Variant of Kunitz-type inhibitor derived from the .alpha.3-chain of human type VI collagen produced by recombinant DNA technology

Brief Summary Paragraph Right (1):

The present invention describes a novel Kunitz type proteinase inhibitor (.alpha.3(VI)-inhibitor) derived from a cDNA of the type VI collagen .alpha.3 chain and peptide variants thereof. In addition a process for preparing the inhibitor and variants thereof by rec. DNA technology as well as pharmaceuticals containing these peptides are described. The .alpha.3(VI) inhibitor variants are characterized by their ability to inhibit serine proteases such as plasma kallikrein and pancreatic and leucocyte elastase.

Brief Summary Paragraph Right (4):

In certain pathological conditions the adequate levels of extracellular proteinase and proteinase inhibitors may be disturbed by excessive release of lysosomal proteinases, such as leucocyte elastase.

Brief Summary Paragraph Right (5):

The consequence may be an extensive proteolytic degradation of connective tissues as well as of humoral proteins including coagulation-, fibrinolysis-, and complements-factors by elastase and other lysosomal proteinases leading to severe clinical symptoms like emphysema, shock lung, ARDS and coagulation disorders.

Brief Summary Paragraph Right (11):

In experimental models of sepsis and emphysema synthetic elastase inhibitors (Powers, Ann. Rev. Respir. Dis. 127, 554-558 (1983)) and natural inhibitors of animal origin such as eglin C (Schnebli et al., Europ. J. Respir. Dis. 66, Suppl. 139, 66-70 (1985) or Hirudin have been shown to be therapeutically useful.

Brief Summary Paragraph Right (17):

Using state-of-the-art rec. DNA technology it was furthermore found that by replacement of one or more of certain aminoacids in and/or around the active center of the .alpha.3(VI) inhibitor specific and potent inhibitors of human plasma kallikrein, pancreatic and leucocyte elastase could be obtained. Therefore, the present invention also relates to synthetic peptide variants of the .alpha.3(VI) inhibitor produced by rec. DNA technology.

Brief Summary Paragraph Right (57):Elastase inhibition assayBrief Summary Paragraph Right (58):

Human leucocyte elastase was obtained from Elastin Products Company Inc., P.O. Box 147, Pacific, Miss., 63069/USA. The substrate used was MeOSuc-Ala-Ala-Pro-Val-pNA (Bachem, Budendorf, Switzerland). The assay conditions are indicated in Table 3. In general, the inhibitor samples were diluted with assay buffer, enzyme was added and the mixture was then preincubated. The reaction was started by addition of substrate (dissolved in DMSO in a concentration of 0,1M and adjusted to the concentration of the stock solution with buffer), and the liberation of p-nitroaniline from the substrate was continuously followed at 405 nm. 100% values were determined in corresponding assays without inhibitors. The inhibition (in percent) was calculated from the following equation.

• Detailed Description Paragraph Right (20):

Selected colonies of the yeast strain SC106 transformed with plasmid-DNA of pKol 100, pKol 101, pKol 102, pKol 103 and pKol 104 were cultivated to shake flasks and 1 L-fermenters under including conditions. Probes of the culture supernatants were tested for trypsin inhibition activity, elastase inhibition activity and plasma kallikrein inhibition activity.



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to a friend**Simplify Coding of Tissue Adhesive Repairs**

Posted on Dec 13, 2001

Tissue adhesives hold many advantages for the general surgeon and patient. They align and seal injured tissue without the use of sutures or staples; promote natural healing; cost less and prove easier and quicker to use than traditional closure methods; and, in some cases, can save the patient a follow-up visit to the doctor. Coding for tissue adhesives poses certain challenges, considering the fact that Medicare and private payers do not share a standard billing procedure for these repairs and such repairs are often reported as part of other integumentary system procedures. Coders should consider the following before coding for tissue adhesives:

Who covers the patient (Medicare or a private payer)?

What other procedures or services were performed?

Who Covers the Patient?

Tissue adhesive repair has had a bumpy coding history since the FDA approved Dermabond (the first and best-known adhesive product) in 1998. CPT did not include tissue adhesives in any of its code descriptors before 2000; an unlisted code was used to report such repairs. CPT 2000 revised the wording in the wound closure section to indicate that "tissue adhesives, either singly or in combination with each other, or in combination with adhesive strips, should be reported using the appropriate existing wound closure code." CPT notes, for example, that if Dermabond is used to close a 3-cm facial laceration that does not require extensive cleansing or removal of particulate matter, the service should be reported using 12013 (simple repair of superficial wounds of face, ears, eyelids, nose, lips and/or mucous membranes; 2.6 cm to 5.0 cm).

Furthermore, CPT did not limit the use of repair codes for tissue adhesive application to "simple" repairs (one layer closure without extensive cleansing or removal of particulate matter). If more extensive closure is required, the appropriate intermediate or complex closure code may be reported even if tissue adhesive is applied to close the skin.

Shortly after CPT 2000 was published, however, CMS introduced G0168 (wound closure utilizing tissue adhesive[s] only) and instructed providers to use this code instead of the existing CPT codes when billing Medicare Part B carriers. The American Medical Association (AMA), the American College of Surgeons (ACS) and other physician groups criticized these guidelines, arguing that the code was introduced without consulting either the AMA or the ACS. The ACS claimed that introducing G0168 was contrary to the CPT 2000 revision, which instructed physicians to use existing codes. The ACS also noted that the reimbursement level for G0168 is about half that for the simple repair codes, because Medicare regards tissue adhesive closure as a simpler technique.

The CMS Final Rule, published in Nov. 1999, noted that the RVUs for G0168 were based on the value of a level 2 established patient visit — 99212 (office or other outpatient visit for the evaluation and management of an established patient, which requires at least two of these three key components: a problem focused history; a problem focused examination; straightforward medical decision making) — with the addition of the price of the Dermabond as a practice expense. CMS states that G0168's lower value was justified because "many of these wounds could have been closed by Steri-strips, a service that is also coded with evaluation and management, rather than a simple repair."

Note: G0168 has 0 global days, which means that CMS will pay for a separate E/M visit if another visit is required for a complication.

Surgeons and their billing staff have had to code tissue adhesive repairs one way for their Part B carrier and another way (or ways) to meet the coding rules of private payers that haven't used HCPCS codes since Jan. 1, 2000, says Susan Callaway,

CPC, CCS-P, a coding and reimbursement specialist and educator in North Augusta, S.C. The existing and appropriate simple, intermediate or complex repair code should be used for most private payers, Callaway says. Some private carriers, however, may still require 17999 (unlisted procedure, skin, mucous membrane and subcutaneous tissue) be submitted for manual review, along with documentation of the procedure. Wellmark (the BC/BS carrier in Iowa and South Dakota) and others recognize G0168.

#### Other Procedures/Services Performed

Many of the same rules that apply to traditional wound closures apply to tissue adhesive repair, regardless of whether the carrier is Medicare or private. If multiple lacerations are repaired, for instance, modifier -59 (distinct procedural service) may need to be appended to indicate the lesion was removed at a different site and should not be bundled or included with the first repair.

If tissue adhesives are used in conjunction with staples or sutures to close a wound, surgeons should report only the appropriate repair code and not bill separately for the tissue adhesive repair, Callaway says. "If, for example, the surgeon sutures the deeper portions of a laceration but uses tissue adhesive to close the skin, only the appropriate complex or intermediate repair code should be reported," Callaway says, adding that G0168 should not be reported separately to Medicare carriers.

G0168 is bundled not only with all repair codes (whether simple, intermediate or complex) but also with many other integumentary and musculoskeletal codes, including:

11055-11057 — paring or cutting of benign hyperkeratotic lesion (e.g., corn or callus)

11100-11101 — biopsy of skin, subcutaneous tissue and/or mucous membrane (including simple closure), unless otherwise listed (separate procedure)

11200 — removal of skin tags, multiple fibrocutaneous tags, any area; up to and including 15 lesions

11300-11303 — shaving of epidermal or dermal lesion, single lesion, trunk, arms or legs

11305-11308 — shaving of epidermal or dermal lesion, single lesion, scalp, neck, hands, feet, genitalia

11310-11313 — shaving of epidermal or dermal lesion, single lesion, face, ears, eyelids, nose, lips, mucous membrane

11010-11012 — debridement including removal of foreign material associated with open fracture(s) and/or dislocation(s)

11040-11044 — debridement.

CCI also bundles G0168 with other integumentary procedures that include closure and with procedures that involve more complex closures.

Although the CCI does not explicitly bundle G0168 with E/M services, many carriers will not reimburse G0168 if it is performed at the same time as an E/M service.

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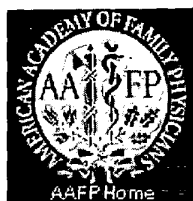
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## Using Tissue Adhesive for Wound Repair: A Practical Guide to Dermabond

THOMAS B. BRUNS, M.D., and  
J. MACK WORTHINGTON, M.D.

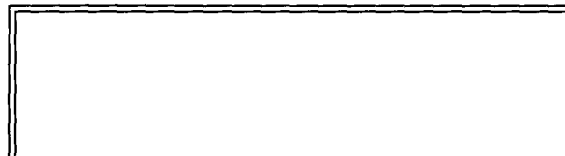
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Dermabond is a cyanoacrylate tissue adhesive that forms a strong bond across apposed wound edges, allowing normal healing to occur below. It is marketed to replace sutures that are 5-0 or smaller in diameter for incisional or laceration repair. This adhesive has been shown to save time during wound repair, to provide a flexible water-resistant protective coating and to eliminate the need for suture removal. The long-term cosmetic outcome with Dermabond is comparable to that of traditional methods of repair. Best suited for small, superficial lacerations, it may also be used with confidence on larger wounds where subcutaneous sutures are needed. This adhesive is relatively easy to use following appropriate wound preparation. Patients, especially children, readily accept the idea of being "glued" over traditional methods of repair. (Am Fam Physician 2000;61:1383-8.)

Physicians have long sought an efficient method of wound repair that requires little time and minimizes discomfort for their patients, yet produces a good cosmetic outcome. Dermabond, the newest tissue adhesive to be labeled by the U.S. Food and Drug Administration (FDA), may well meet those criteria. Cyanoacrylate tissue adhesives have been around for more than 20 years but have only recently become available for use in this country for incisional and laceration repair. They have been shown to have negligible histotoxicity, to form a strong bond to wound edges and to provide long-term cosmesis equivalent to, or better than, traditional methods of repair. In addition, they require less than one half the time for wound closure. *Table 1* summarizes the benefits of tissue adhesive over sutures.<sup>1-11</sup>

### History and Characteristics of Cyanoacrylates

Cyanoacrylate tissue adhesives combine cyanoacetate and formaldehyde in a heat vacuum along with a base to form a liquid monomer.<sup>9</sup> When the monomer comes into



contact with moisture on the skin's surface, it chemically changes into a polymer that binds to the top epithelial layer. This polymer forms a cyanoacrylate bridge, binding the two wound edges together and allowing normal healing to occur below. The conversion from monomer to polymer occurs rapidly, preventing seepage of the adhesive below the wound margins as long as the edges are well apposed. Heat is often generated during the change from monomer to polymer, and this heat may be felt on occasion by patients during application to the skin. Cyanoacrylates have also been shown to have antimicrobial properties.<sup>10-14</sup>

Cyanoacrylates were first manufactured in 1949. The first adhesives were noted to have extreme inflammatory effects on tissues. *N*-butyl-2-cyanoacrylate, which was developed in the 1970s, was the first adhesive to have negligible tissue toxicity and good bonding strength, as well as acceptable wound cosmesis.

*N*-butyl-2-cyanoacrylate has been used in cartilage and bone grafting, coating of corneal ulcers in ophthalmology, repair of damaged ossicles in otolaryngology, coating of aphthous ulcers, embolization of gastrointestinal varices and embolization in neurovascular surgery.<sup>1,2,8,10,11</sup> This adhesive is not labeled for this use by the FDA but has been used in Canada and numerous other countries for more than 20 years.

Dermabond (2-octylcyanoacrylate), the latest in cyanoacrylate technology, has less toxicity and almost four times the strength of *N*-butyl-2-cyanoacrylate.<sup>15</sup> Special plasticizers have been added to the formula to provide flexibility. This adhesive reaches maximum bonding strength within two and one-half minutes and is equivalent in strength to healed tissue at seven days post repair.<sup>15</sup>

### TABLE 1 Advantages of Adhesive vs. Sutures

<p>Maximum bonding strength at two and one-half minutes Equivalent in strength to healed tissue at seven days post repair Can be applied using only a topical anesthetic, no needles Faster repair time Better acceptance by patients Water-resistant covering Does not require removal of sutures</p>
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<p>In the repair of small wounds, Dermabond provides results equivalent to traditional closure methods and application requires less than one half the time.</p>
--

### When Can Dermabond Be Used?

Dermabond is marketed as a replacement for sutures that are 5-0 or smaller in diameter. Properly selected wounds on the face, extremities and torso may be closed with the adhesive. The use of adhesive rather than sutures is solely up to the discretion of the physician and will reflect his or her level of comfort and experience. Extremity and torso wounds tend to heal better when subcutaneous sutures are placed first. If adhesive is chosen by the physician to be used on areas of high tension or mobility (such as joints), this area should be immobilized in a splint to prevent premature peeling of the adhesive.

Scalp wounds may be closed with adhesive using meticulous care so as not to allow excess

adhesive to run through the hair. Dermabond must be kept dry in this area for at least five days for normal healing.

This tissue adhesive should not be used on animal bites, severely contaminated wounds, ulcers, puncture wounds, mucous membranes (including mucocutaneous junctions) or areas of high moisture content, such as the groin or axillae (Table 2). The adhesive may be used on selected hand, foot and joint wounds if these areas are kept dry and immobilized.<sup>4-6</sup>

Dermabond reaches maximum bonding strength in two and one-half minutes and is equal in strength to healed tissue seven days after traditional closure with sutures.

## Technique for Use in Wound Closure

The availability of a tissue adhesive by no means obviates the need for thorough wound irrigation and cleansing. Deeper wounds should undergo thorough wound preparation as with traditional methods of repair to reduce the risk of infection. This will often include the need for topical or local anesthesia. Good wound management should not be compromised for a quick repair with a tissue adhesive.

**TABLE 2**  
Contraindications to Use of Skin Adhesives

Jagged or stellate lacerations  
Bites, punctures or crush wounds  
Contaminated wounds  
Mucosal surfaces  
Axillae and perineum (high-moisture areas)  
Hands, feet and joints (unless kept dry and immobilized)

Wound closure with Dermabond is achieved in several steps (Table 3). Smaller lacerations can often be cleansed with an antibacterial compound and flushed with sterile saline solution before closure. Small lacerations on the face usually heal well with this preparation. One study<sup>6</sup> showed that only one of five children needed local anesthesia for repair of minor

facial lacerations with Dermabond. Because the adhesive peels off in five to 10 days, deeper lacerations to the torso and extremities should have subcutaneous sutures placed to strengthen the wound closure and optimize long-term cosmesis. Deep wounds without subcutaneous sutures seem to have a higher dehiscence rate.<sup>4</sup>

Dermabond comes in a single-use vial in sterile packaging. It consists of an outside plastic casing with an inner glass ampule containing 0.5 mL of adhesive that can be expressed through the applicator tip once the vial has been crushed. As the adhesive moves through the applicator tip, it mixes with an initiator and begins the chemical change from monomer to polymer. Moisture on the skin's surface adds the final catalyst to create the strong polymer bond that bridges the wound edges.

**TABLE 3**

After cleansing, the wound should be positioned so that excess adhesive does not run off into areas not meant to be glued. If this should occur, the excess adhesive should

### Steps in Use of Dermabond

1. Apply topical anesthetic as needed.
2. Prepare wound with antiseptic.
3. Appose wound edges.
4. Crush Dermabond vial and invert.
5. Gently brush adhesive over laceration.
6. Avoid pushing adhesive into wound.
7. Apply three layers of adhesive.

quickly be wiped away with a dry gauze. Good hemostasis should be achieved using pressure on the wound or application of 1:1,000 topical epinephrine solution, if needed. On facial wounds, prior application of a topical anesthetic with epinephrine is usually sufficient. Excessive wound seepage before closure may prevent good bonding to the epithelial layer and may also result in excessive heat production during polymerization.

The edges of the wound must be approximated manually and evenly. If there is uncertainty about whether this can be done, the wound should probably be sutured instead. Forceps or manufactured skin approximation devices may also be used, if preferred. Lack of wound edge eversion does not seem to alter long-term cosmetic outcome.<sup>4-7</sup>

Once the edges have been approximated, the Dermabond vial is crushed between the thumb and index finger and inverted. The vial must be used in the next few minutes or polymerization in the applicator tip will prevent expression of the adhesive. The adhesive is expressed by gently squeezing the vial, which allows the adhesive to be seen at the applicator tip. If the vial is squeezed too hard, adhesive may drip from the end of the vial. To prevent this from happening, the vial must be squeezed gently and squeezing must stop when a drop begins to form at the tip, allowing the adhesive to be drawn back up into the vial with the vacuum thus created.

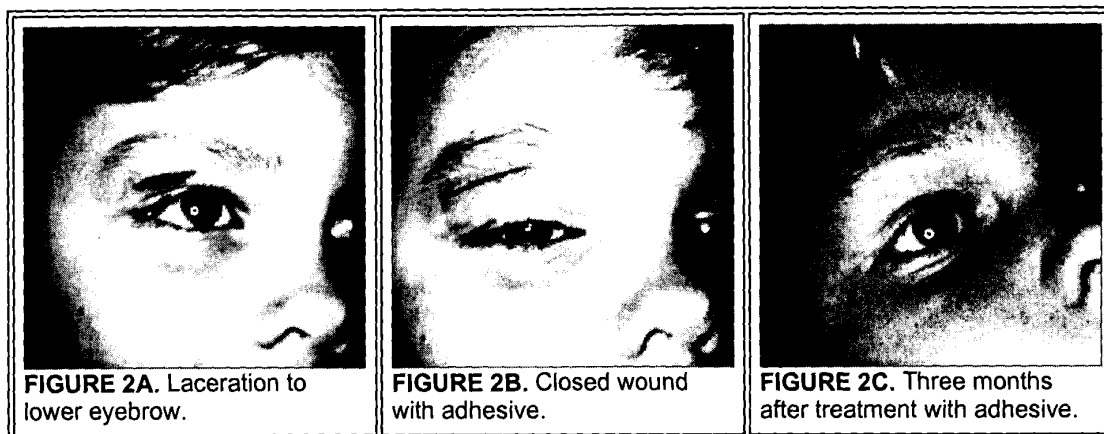
Once the adhesive is at the applicator tip, it is applied to the apposed wound edges with gentle brushing motions. At no time should the applicator tip be pressed into the wound; this may cause adhesive to enter the wound, which may lead to a foreign-body reaction and prevent normal wound healing or cause dehiscence.<sup>4</sup> Adhesive will not enter the wound unless it is placed there by force. After applying adhesive across the wound edges and holding the edges together for at least 30 seconds before releasing, more adhesive should be applied in an oval pattern around the wound to encompass a greater surface area on the skin--this adds greater strength to the wound closure (*Figure 1*). At least three layers should be applied to ensure optimal strength to the wound closure. The first layer of adhesive reaches maximal strength within two and one-half minutes; the subsequent layers usually take longer to dry because less moisture is available for polymer formation. The wound should not be touched until the adhesive dries completely. Fanning or blowing on the wound will not speed up polymerization.



**FIGURE 1.** Application of adhesive to chin.

The adhesive acts as its own water-resistant bandage, and no added coverings are needed. Patients may shower normally and pat the area dry. The adhesive will spontaneously peel off in five to 10 days. No topical antibiotics should be applied to the closed wound because this would break down the adhesive and cause early peeling. In active children, a bandage may be recommended to prevent them from picking at their wound or reinjuring themselves in the same location. Children should not take baths, because excessive exposure to water

may loosen the top epithelial layer of skin and cause premature peeling or wound dehiscence. Examples of facial wounds repaired with Dermabond are shown in *Figures 2a, 2b, 2c, 3a and 3b*.



## Precautions

Randomized controlled clinical trials<sup>4-8</sup> have shown that infection rates are not significantly different between wounds that have been sutured and wounds that have been closed with Dermabond. However, if adequate wound cleansing and preparation are compromised because of the ease of use of a tissue adhesive, an increase in infection rates could occur.

Suspected infection below the adhesive may be treated with oral antibiotics. Purulence from a true infection generally pushes the dried polymer away from the skin. In these rare cases, the adhesive should be removed and standard wound care measures should be initiated. Reapplication of adhesive in such cases is not recommended.

Dermabond adhesive acts as its own water-resistant bandage, and no other coverings are needed.

Application of adhesive to a wound will sometimes result in run-off to areas not intended to be glued. Excess adhesive can simply be wiped away with a dry gauze if done immediately. If an object such as a finger or forceps becomes inadvertently adhered to the patient during repair, place pressure on the patient's skin adjacent to the edge of the object and gently roll the object away. This action allows the object to be peeled away from the skin without pulling the edges of the wound apart.

If wound edges are not aligned after the first application of adhesive, wipe the glue away immediately with a dry gauze. A 10-second "grace period" exists before the adhesive becomes too polymerized to wipe away. If the adhesive has already dried, the application of antibiotic ointment or petroleum jelly for 30 minutes will loosen the polymer for removal.

Generous amounts of ophthalmic antibiotic ointment may be placed on eyelids that have been inadvertently glued shut. Lids should not be pried open or eyelashes removed, as the lids will open after one to two days with this method.

Cyanoacrylates are used for corneal perforations and are not harmful to the eye.<sup>13</sup> The



placement of gauze over the eyes when working in this area should prevent exposure to run-off.

Repeat trauma to a wound or excessive picking at the wound may result in dehiscence. Reported rates of wound dehiscence are extremely low, ranging from three out of 68 patients in one study to no cases of dehiscence in two studies with 96 patients combined.<sup>4-8</sup> Depending on the time of presentation, these wounds could be closed again with adhesive or sutures.

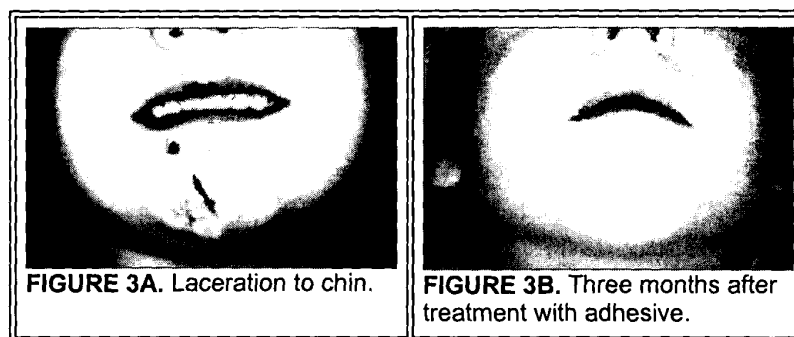


FIGURE 3A. Laceration to chin.

FIGURE 3B. Three months after treatment with adhesive.

## Cost

Dermabond is the only FDA-labeled and commercially available adhesive in this country and costs approximately \$24 a vial (12 vials per box), with a shelf-life of two years. Sutures commonly used in the ambulatory care setting generally cost about \$5 per package. In most cases when Dermabond is used, a suture tray need not be opened for minor facial lacerations, because only gauze, antiseptic solution, sterile saline and tissue adhesive are needed for closure. Patients can be quickly treated with this method. All other closures will require suture equipment and local anesthesia to ensure painless wound preparation and placement of deep sutures. Dermabond saves time even when used after placement of subcutaneous sutures and requires no suture removal or follow-up visit. Unless a complication develops, wounds closed with tissue adhesive need not be seen again.

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On the other hand, the inhibition constant of Guamerin which was obtained by employing elastase as a substrate was determined as  $8.1 \times 10^{-14}$  M, which was considerably low compared with those of other elastase-inhibiting proteins in the prior art, such as eglin C ( $10^{-10}$  -  $10^{-11}$  M) isolated from human skin or elafin ( $6 \times 10^{-10}$  M) isolated from european leeches. As a result, it is found that Guamerin is of a higher inhibition activity than any other elastase-inhibiting proteins known in the art. Moreover, as shown in FIG. 5, it was clearly confirmed that: Guamerin inhibits the elastase activity in a competitive manner, since Guamerin competes with the substrate for elastase in the binding to the target enzyme, i.e., elastase.